Application Note 24

Distinguishing Regioisomers in Pharmaceutical Products using Benchtop NMR Spectroscopy

Introduction

NMR spectroscopy is an incredibly powerful technique for the characterisation and analysis of many chemical compounds. Unlike many other analytical techniques, it is straight-forward to distinguish subtle differences in chemical structures. Isomers, compounds with the same molecular formula but different structures, can have varying chemical and physical properties. Understanding isomerism helps introduce safer and more effective drugs and their alternatives. Benchtop NMR spectroscopy distinguishes even between regioisomers, which differ only by the substitution patterns about an aromatic ring.

In this application note, both one-dimensional (^{1}H, ^{13}C) and two dimensional (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC) spectra obtained on the **Oxford Instruments X-Pulse Broadband Benchtop NMR Spectrometer** are analysed. The differences in the spectra, particularly the aromatic regions, are used to distinguish the three regioisomers of hydroxyacetanilide (*ortho*-, *meta*- & *para*-), demonstrating the ease of differentiating regioisomers of small organic molecules.

The most common isomer, *para*-hydroxy-acetanilide (known as paracetamol or acetaminophen), is regarded as a first line of defence against pain by the World Health Organisation. Paracetamol also has additional antipyretic (fever prevention and reduction) benefits, as well as the ability to combat allergic symptoms when combined with other drugs. *Ortho*-hydroxyacetanilide also has a variety of uses including as an anti-inflammatory and antirheumatic drug; and while *meta*-hydroxyacetanilide has yet to been marketed as a drug, it does possess analgesic (pain relieving) properties.

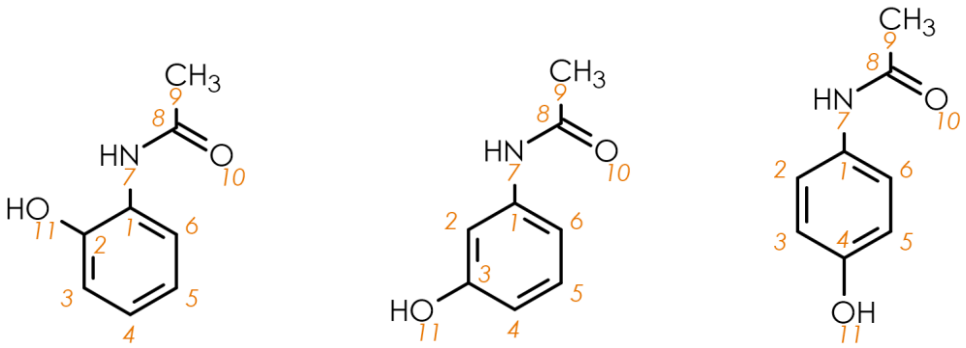

Figure 1 Molecular structures for the hydroxyacetanilide isomers, *ortho* (left), *meta* (centre), and *para* (right).

Hydroxyacetanilides

Hydroxyacetanilides consists of acetamido {–NHC(O)CH_3} and hydroxy (–OH) groups substituted on an aromatic ring. The various relative substitution positions of two groups around the aromatic ring, rise to three regioisomers: *ortho*-, *meta*- and *para*- (*Figure 1*). Each isomer can readily be distinguished by NMR spectroscopy, specifically due to the differences in the aromatic regions (δ_H 6 - 8 ppm, δ_C 110 - 170 ppm) arising from the different substitution patterns.

By inspecting the molecular structures, seven chemically unique hydrogen environments, and eight chemically unique carbon environments can be identified in both the *ortho*- and *meta*- isomers. While due to the symmetry around the aromatic ring in the *para*- isomer (2 & 6, and 3 & 5 are chemically equivalent), there are only five hydrogen environments, and six carbon environments. Since each chemically unique environment is expected to give a single signal in an NMR spectrum, this is the first indication how these isomers could be distinguished.

Proton (^{1}H) NMR

The proton NMR spectra of the three hydroxyacetanilides are shown in *Figure* 2. In all three cases the methyl group 9-CH_3 gives a singlet at δ_H 2 ppm, while the hydroxy *11*-OH and amine *7*-NH hydrogen give broad signals around δ_H 9 - 10 ppm. The compounds can be distinguished by the remaining aromatic signals in the range δ_H 6 - 8 ppm.

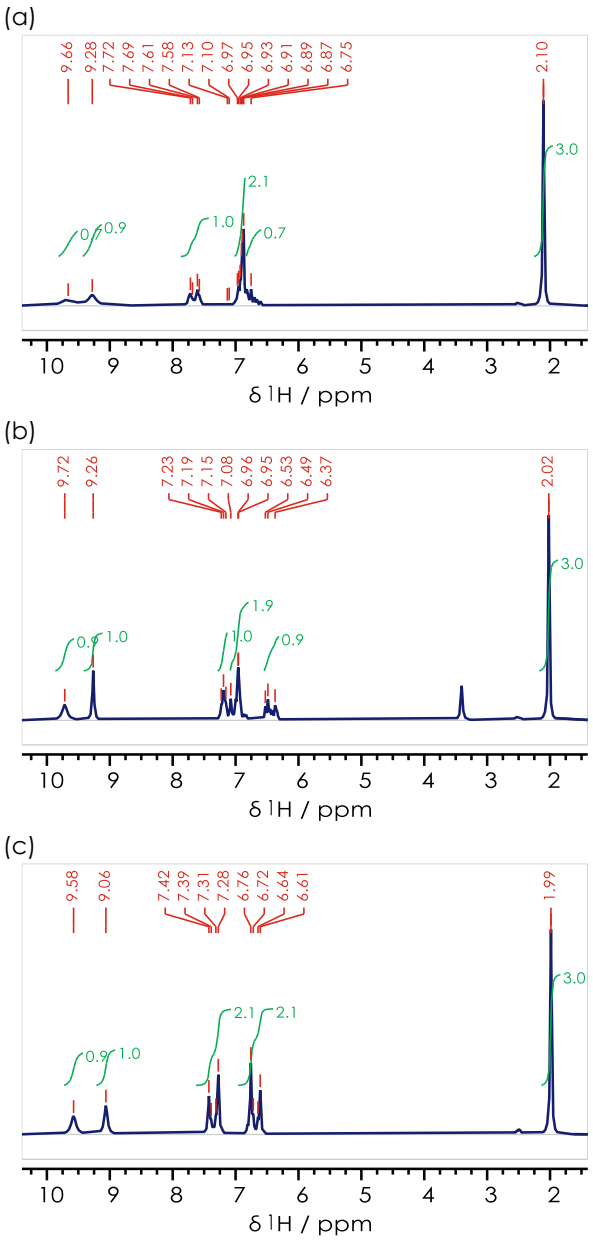

Figure 2 ^{1}H spectra for the (a) *ortho*- (b) *meta*- (c) *para*- isomers of hydroxyacetanilide in DMSO-d_6

Each chemically unique hydrogen environment will give a single signal in the ^{1}H NMR spectrum. These signals may be made up of one or more individual peaks, with the number and relative intensities depending on the other nearby hydrogen atoms; in most cases 'nearby' means separated by at most three chemical bonds, which usually would be H–C–C–H. For example, considering *meta*-hydroxyacetanilide, 4-, 5- & 6-CH are close enough together for each signal to comprise multiple peaks; while 2-CH is far enough from any other hydrogen atoms so should give a signal with only one peak. These signals are all present but overlapped in the aromatic region of the ^{1}H NMR spectrum (*Figure 2b*).[1] The same arguments can be used for *ortho*-hydroxyacetanilide where all four aromatic hydrogens would be expected to give rise to signals with multiple peaks, which overlap at benchtop frequencies. While for *para*-hydroxyacetanilide, due to the molecular symmetry there's two signals each of which include two main peaks arising from a single three-bond interaction (e.g., 2-H–C–C–H-3). This allows for *para*-hydroxyacetanilide (paracetamol, *Figure 2c*) to be readily distinguished from the other regioisomers.

Due to the signal overlap, to unequivocally assign all three regioisomers by NMR spectroscopy, more information is required in addition to that obtained from a simple one-dimensional proton spectrum.

Carbon-13 (^{13}C) NMR

One option is to look at one-dimensional carbon-13 spectra, since these are obtained proton decoupled, each chemical environment gives a single peak in the NMR spectrum (*Figure 3*). For all three compounds the methyl group 9-CH_3 gives a signal at δ_C 23 ppm, while 8-CO gives a peak at δ_C 168 ppm, and the aromatic carbons 1-6 give signals over the range 160 – 105 ppm.

As with the proton NMR spectrum, *para*-hydroxyacetanilide can easily be identified, since due to its symmetry there's only four unique chemical environments in the aromatic ring (1-C, 2/6-CH, 3/5-CH, 4-C), and hence four aromatic signals in the ^{13}C{^{1}H} NMR spectrum (*Figure 3c*).

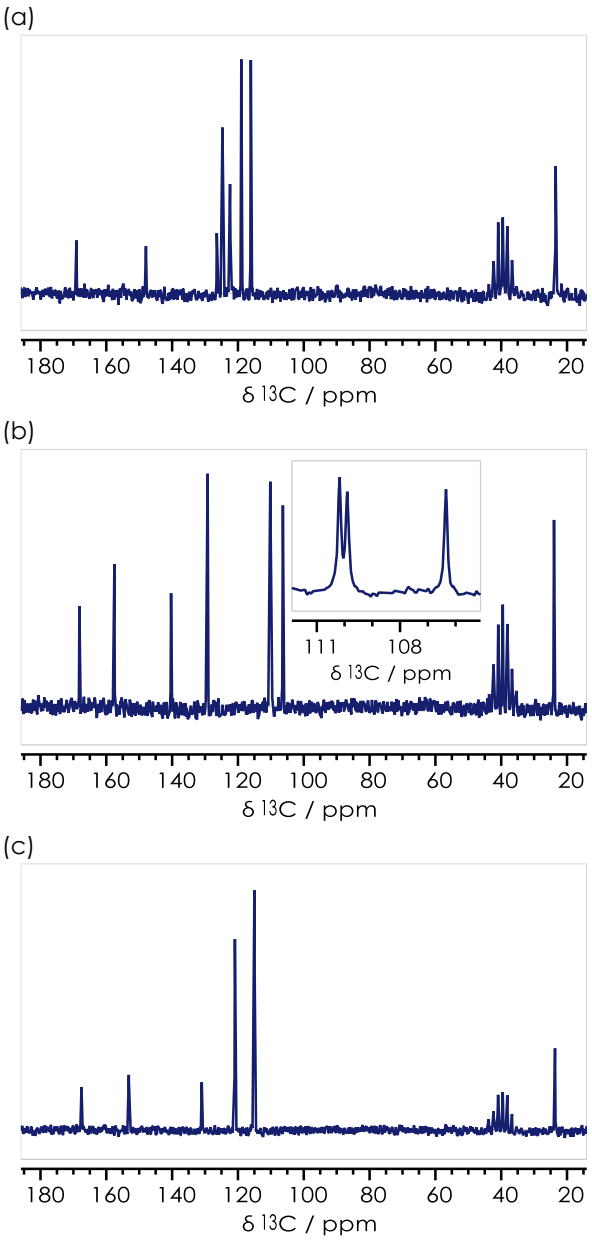

Figure 3 ^{13}C {^{1}H} spectra for the (a) *ortho*- (b) *meta*- (c) *para*- isomers of hydroxyacetanilide in DMSO-d_6

[1] To increase the signal dispersion in the ^{1}H NMR spectrum, so each signal can be clearly distinguished, requires use of a *significantly* higher magnetic field strength than can be obtained from benchtop instruments using permanent magnets.

Both *ortho-* (*Figure 3a*) and *meta-* hydroxyacetanilide (*Figure 3b*) have six distinct aromatic signals. While it *may* be possible to assign each of these signals to the appropriate aromatic carbon, using a theoretical understanding of the origin of chemical shifts; doing so is non-trivial beyond the scope of this application note.

Two-Dimensional Spectra

A good amount of structural information can be interpreted from the one-dimensional ^{1}H and ^{13}C NMR spectra. However, by obtaining two-dimensional NMR spectra, the signal dispersion of the ^{13}C spectra, and the information content of the ^{1}H spectra can be combined; to give even more structural information, thereby unequivocally distinguishing the three regioisomers.

^{1}H-^{13}C Correlation Spectra

In a two-dimensional ^{1}H-^{13}C HSQC (**H**eteronuclear **S**ingle **Q**uantum **C**oherence) spectrum; one-dimensional ^{1}H and ^{13}C spectra are shown along the *x-* and *y*-axis respectively; and the cross peaks correspond to one bond ^{1}H-^{13}C interactions. Therefore, allowing us to match up any ^{13}C environments to their corresponding ^{1}H environments.

The aromatic regions of the ^{1}H-^{13}C HSQC spectra for the three hydroxyacetanilide regioisomers are shown in *Figure 4*. In all three cases by showing the data in two-dimensions, each signal is clearly resolved, and the three regioisomers can be easily identified and distinguished.

Once again, the easiest isomer to identify is *para*-hydroxyacetanilide (*Figure 4c*), where the two signals are clearly distinguished, and appear as two peaks.[2] Whereas for both the *ortho-* (*Figure 4a*) and *meta-* (*Figure 4b*) isomer, four signal are clearly observed in the two-dimensional HSQC spectrum. These can be distinguished by the presence of a signal comprising a single sharp peak for *meta*-hydroxyacetanilide (δ_H + 6.96, δ_C +109.9 ppm), which corresponds to 2-CH; while all the other signals in the HSQC show clear broadening (or multiple peaks) in the ^{1}H dimension, due to interactions between adjacent CH groups in the molecules.

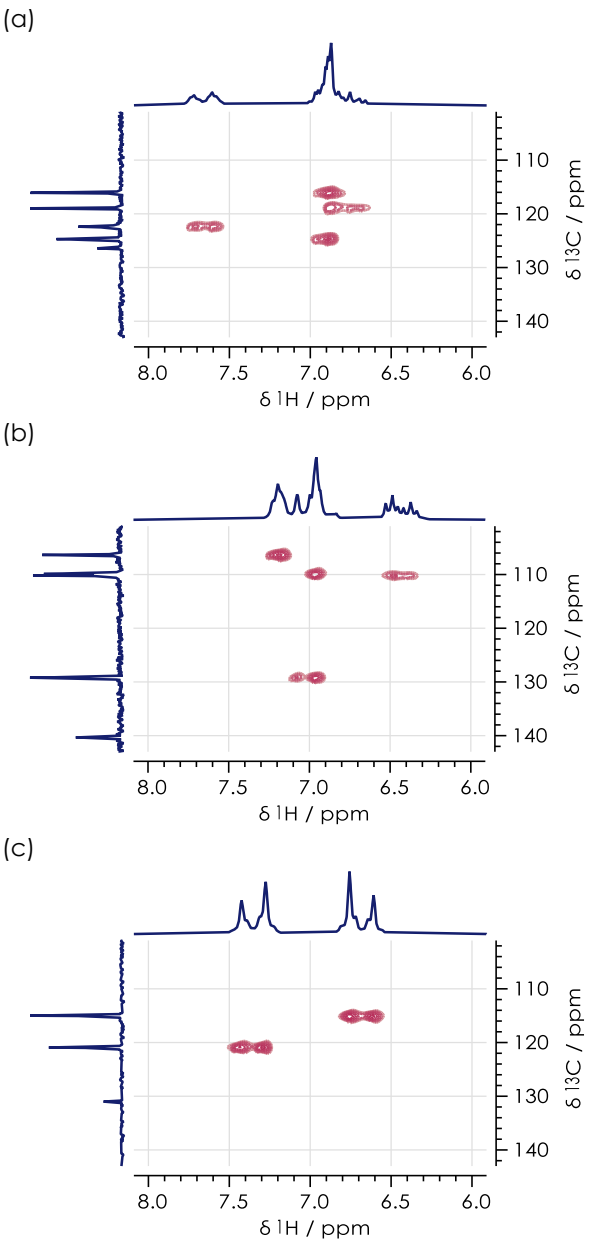

Figure 4 aromatic regions of ^{1}H-^{13}C HSQC spectra for the (a) *ortho-* (b) *meta-* (c) *para-* isomers of hydroxyacetanilide in DMSO-d_6

[2] Corresponding to the two main peaks for each signal in the ^{1}H NMR spectrum.

^{1}H-^{1}H Correlation Spectra

Another type of two-dimensional NMR spectrum which *could* be used to distinguish regioisomers, is a ^{1}H-^{1}H COSY (**Co**rrelation **S**pectroscop**y**) spectrum. Where off-diagonal signals are present in the spectrum when there's coupling between ^{1}H NMR signals (which gives rise to signals with multiple peaks in the one-dimensional spectrum).[3]

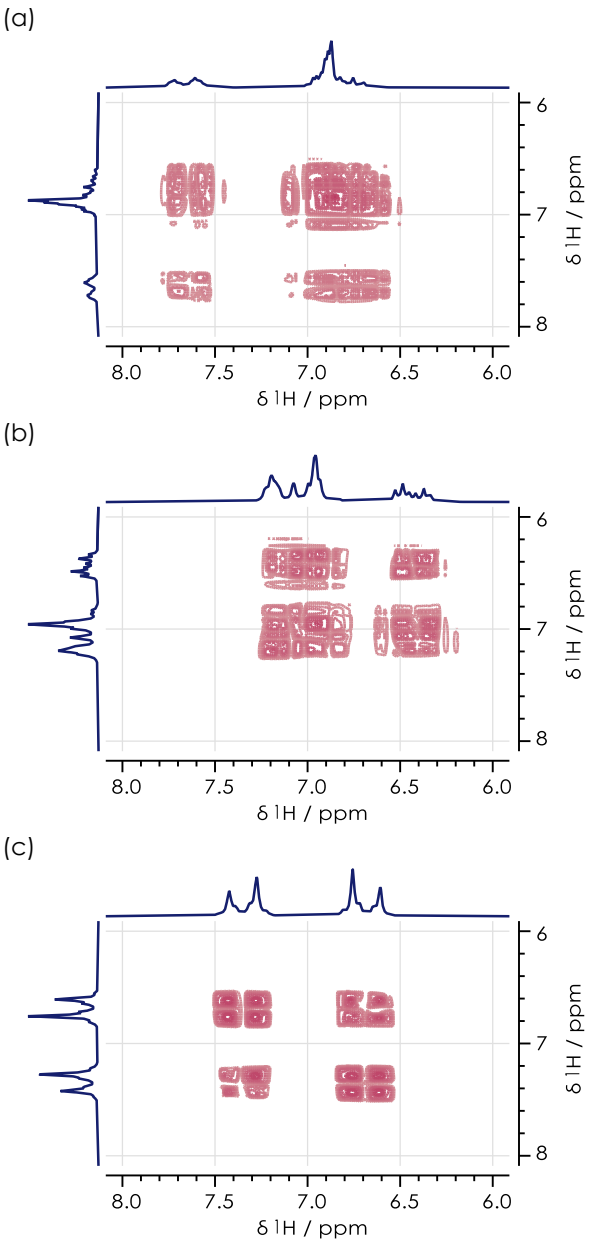

Figure 5 aromatic regions of ^{1}H-^{1}H COSY spectra for the (a) *ortho-* (b) *meta-* (c) *para-* isomers of hydroxyacetanilide in DMSO-d_6

The aromatic regions of the ^{1}H-^{1}H COSY spectra for the three hydroxyacetanilide regioisomers are shown in *Figure 5*. Unfortunately, in this case, due to the significant amount of signal overlap, they cannot be reliably distinguished.

Spectroscopic Data

The fully assigned spectra data of the three regoisomers of hydroxyacetanilide, is as follows.

***ortho*-hydroxyacetanilide**

^{1}H NMR (60 MHz, DMSO-d_6, +40°C) δ_H 9.66 (br s, 1H, *7*-NH), 9.28 (br s, 1H, *11*-OH), 7.80 – 7.48 (m, 1H, 6-CH), 7.17 – 6.53 (m, 3H, *3*,*4*,*5*-CH), 2.10 (s, 3H, 9-CH_3); ^{13}C{^{1}H} NMR (15 MHz, DMSO-d_6) δ_C 169.08 (*8*-CO), 147.96 (*2*-C), 126.42 (*1*-C), 124.71 (*4*-CH), 122.40 (6-CH), 118.98 (*5*-CH), 116.09 (*3*-CH), 23.45 (9-CH_3).

***meta*-hydroxyacetanilide**

^{1}H NMR (60 MHz, DMSO-d_6, +40°C) δ_H 9.72 (br s, 1H, *7*-NH), 9.26 (br s, 1H, *11*-OH), 7.31 – 6.74 (m, 3H, *2*,*5*,6-CH), 6.60 – 6.26 (m, 1H, *4*-CH), 2.02 (s, 3H, 9-CH_3); ^{13}C{^{1}H} NMR (15 MHz, DMSO-d_6) δ_C 168.17 (*8*-CO), 157.55 (*3*-C), 140.32 (*1*-C), 129.21 (*5*-CH), 110.18 (*4*/6-C), 109.90 (*2*-CH), 106.35 (6/*4*-C), 23.96 (9-CH_3).

***para*-hydroxyacetanilide**

^{1}H NMR (60 MHz, DMSO-d_6, +40°C) δ_H 9.58 (br s, 1H, *7*-NH), 9.06 (br s, 1H, *7*-NH), 7.54 – 7.15 (m, 2H, *2*,6-CH), 6.88 – 6.48 (m, 2H, *3*,*5*-CH), 1.99 (s, 3H, 9-CH_3); ^{13}C{^{1}H} NMR (15 MHz, DMSO-d_6) δ_C 167.53 (*8*-CO), 153.16 (*4*-C), 130.99 (*1*-C), 120.93 (*2*,6-CH), 115.00 (*3*,*5*-CH), 23.65 (9-CH_3).

[3] See Application Notes 7 and 21 for further details.

Summary

The chemical structures of the three regioisomers: *ortho-*, *meta-* and *para-* hydroxyacetanilide, were elucidated using a combination of one- and two-dimensional NMR spectra obtained by the **Oxford Instruments X-Pulse Broadband Benchtop NMR Spectrometer**. From these spectra we can clearly differentiate between the three isomers of the same small organic molecule hydroxyacetanilide that contributes to the pain and fever relieving benefits of paracetamol.

The **Oxford Instruments X-Pulse Broadband Benchtop NMR Spectrometer** is avaliable with a comprehensive range of one- and two-dimensional characterisaiton sequences and analyses as as standard. An optional 25 position autosampler can be used to maximise efficiency and throughput; for example, perfrormig screening studies to identify pharmaceutically relevant target molecules.

If you have any questions about this application note, please contact our experts:
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